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Automated Analysis of Glutathione Peroxidase, S-Transferase, and Reductase Activity in Animal Tissue

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JOURNAL ARTICLE

An Automated Analysis of Glutathione Peroxidase, S-Transferase, and Reductase Activity in Animal Tissue

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Abstract

A centrifugal analyzer and a spectrophotometer were compared for routine analysis of xenobiotic metabolizing enzymes glutathione (GSH) peroxidase, GSH-S-transferase, and GSH reductase. Lung, liver, and kidney from 60-day-old male rats were used as the source of enzymes. Linear regression analysis was used to assess the accuracy and precision of the centrifugal analyzer method in measuring enzyme activities. Biologically and statistically, the centrifugal analyzer proved to be acceptable for routine measurement of these GSH-dependent enzymes.

Introduction

Due to the importance of the enzymes glutathione (GSH) peroxidase, GSH-S-transferase, and GSH reductase in xenobiotic metabolism and their sensitivity to known airborne pollutants (oxidants and heavy metals) they should be included in the evaluation of health effects due to inhalation of various environmental toxicants. Assays for these GSH-dependent enzymes were adapted to the centrifugal analyzer so that increased sample numbers per assay could be analyzed efficiently without compromising the reproducibility and accuracy of the results.

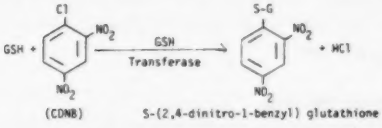
GSH peroxidase (EC 1.11.1.9) catalyzes the breakdown of hydrogen peroxide (H_2O_2) and the reduction of organic hydroperoxides formed from unsaturated fatty acids (1). GSH peroxidase is measured indirectly by a coupled reaction with GSH reductase. The product of GSH peroxidase, the oxidized form of GSH (GSSG), acts as a substrate for GSH reductase, as shown in Equations 1 and 2.

$$2\ GSH + ROOH \xrightarrow[\text{peroxidase}]{GSH} GSSG + ROH + H_2O \quad \text{Eq. 1}$$

$$GSSG + NADPH + H^+ \xrightarrow[\text{reductase}]{GSH} 2\ GSH + NADP^+ \quad \text{Eq. 2}$$

In xenobiotic metabolism, GSH acts as a substrate for all fractions of GSH-S-transferase (EC 2.5.1.18). The nucleophilic GSH and electrophilic foreign substrate bind to the transferase forming a less toxic GSH conjugate. Previous spectrophotometric methods required individual substrates for specific transferases. However, Habig (2) showed 1-chloro-2,4-dinitrobenzene (CDNB) to be active in all fractions, thereby providing a convenient and sensitive assay for GSH-S-transferase (Eq. 3).

Glutathione reductase (EC 1.6.4.2) catalyses the reduction of GSSG by NADPH (Eq. 2). The primary role of GSH reductase is to replenish the levels of reduced GSH that have been oxidized by free radicals, peroxides, oxygen, or enzymatic pathways such as GSH peroxidase. The maintenance of the intracellular pool of GSH by GSH reductase is essential in the metabolism of xenobiotic compounds.



(CDNB) S-(2,4-dinitro-1-benzyl) glutathione

Eq. 3

Materials

A Centrifichem System 400 centrifugal analyzer (Union Carbide) and Beckman Model 25 spectrophotometer were used in the study. Potassium phosphate and potassium chloride were purchased from Fisher Scientific. *Tris* (hydroxy methyl) aminoethane, disodium ethylenediaminetetraacetate (EDTA), glutathione reductase, 1-chloro-2,4-dinitrobenzene, and oxidized glutathione (GSSG) were supplied by Sigma Chemical Co. Potassium cyanide (KCN) and cumene hydroperoxide (ROOH) were obtained from Matheson Coleman and Bell.

Buffers and Reagents

Working reagent for GSH peroxidase was prepared by mixing 32 mL of 100 mmol/L potassium phosphate pH 7.6; 4 mL of 1.33 mmol/L NADPH in distilled water; 4 mL of 3.75 mmol/L sodium azide, 30 mmol/L EDTA and 10 mmol/L KCN in distilled water; 30.6 mg of GSH; and 40 units of GSH reductase (228 units/mL).

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Working reagent for GSH-S-transferase was prepared by mixing 39.6 mL of 100 mmol/L potassium phosphate pH 6.5; 0.4 mL of 25 mmol/L CDNB in 95% ethanol; and 12.4 mg of GSH.

Working reagent for GSH reductase was prepared by mixing 32 mL of 100 mmol/L potassium phosphate and 3 mmol/L EDTA pH 7.0; 4 mL of 1 mmol/L NADPH in distilled water; and 4 mL of 15 mmol/L oxidized glutathione in distilled water.

During experimentation, the addition of EDTA to the assays for GSH peroxidase and reductase was essential when using spectrophotometers with stainless steel automated samplers or the Centrifichem 400 with a stainless steel rotor. Elimination of EDTA results in a rapid decrease in enzyme activity, probably due to leaching of metal cations from the stainless steel. However, if glass cuvettes are used in the spectrophotometer, the elimination of EDTA has no observable effect on enzyme activity.

Methods

Sample Preparation

Lung, liver, and kidney were removed from twelve 60-day-old Charles River CD Sprague Dawley rats sacrificed by cervical dislocation, and homogenized 1:5 (w:v), 1:4 (w:v), and 1:5 (w:v) in ice-cold 50 mmol/L *Tris*-HCL, 200 mmol/L potassium chloride pH 7.6, respectively. All of the following supernatants were diluted with the same *Tris* buffer as above. The homogenates were centrifuged at 10,000 × g for 15 minutes at 4°C, excess fat was removed, and supernatants decanted and recentrifuged at 100,000 × g for 60 minutes at 4°C. The resultant lung supernatants were diluted 1:10 for all enzyme activities. The liver supernatants were diluted 1:60 for reductase activity, and 1:120 for peroxidase and transferase activities. The kidney supernatants were diluted 1:20 for reductase and transferase activities and 1:60 for peroxidase activity. All diluted samples were divided into two equal aliquots to be comparatively assayed by the spectrophotometer and centrifugal analyzer. The samples were stored at -80°C until used.

The assays for GSH peroxidase, GSH-S-transferase, and GSH reductase are modifications of methods previously reported (3-5), respectively.

Centrifichem Methods

For GSH peroxidase, the centrifugal analyzer was set at: filter 340 nm, temperature 30°C, hold blank, rate, time delay 15 seconds, time interval 1 minute, concentration factor 0000, abnormal absorbance 0.0L, number of prints 4, test code 00, and print out absorbance. Sample cups #0 to 29 were filled with 7 mmol cumene hydroperoxide (ROOH) in 50% EtOH. A 5-μL aliquot of diluted tissue samples was hand pipetted into reagent well #2 on the disc. The pipettor was set to deliver 10 μL of sample (ROOH), 10 μL of sample plus diluent, and 350 μL of reagent.

The settings for GSH-S-transferase were: filter 340 nm, temperature 30°C, auto blank, rate, time delay 30 seconds, time interval 1 minute, concentration factor 0000, abnormal absorbance 1.OU, number of prints 4, test code 00, and printout absorbance. The pipettor was set to deliver 30 μL of lung sample, 40 μL of sample plus diluent (deionized water), and 350 μL reagent; 5 μL of liver sample, 50 μL of sample plus diluent, and 350 μL of reagent; and 20 μL of kidney sample, 30 μL of sample plus diluent, and 350 μL of reagent.

The analyzer settings for GSH reductase were the same as those for GSH-S-transferase, except for abnormal absorbance at 0.0L. The pipettor was set to deliver 50 μL of lung sample, 60 μL of sample plus diluent, and 350 μL of reagent; 25 μL of liver sample, 35 μL of sample plus diluent, and 350 μL of reagent; and 10 μL of kidney sample, 20 μL of sample plus diluent, and 350 μL of reagent.

Manual Method

All reagents and volumes were the same as those used for the corresponding Centrifichem method. A glass microcuvette was used so that the total reaction volume (*i.e.*, sample, diluent, and reagent volumes) used in the Centrifichem method could also be run on the spectrophotometer. The enzyme reactions were carried out at 30°C in a thermostatically-controlled cuvette holder. The reaction was run for 5 minutes with absorbance readings taken every minute. The absorbance readings were averaged and the mean taken to determine absorbance/min. In determining glutathione peroxidase activity, a non-enzymatic rate of NADPH oxidation was subtracted from all experimental samples.

Protein and Enzyme Determination

Standard curves of crystalline bovine serum albumin were obtained by both methods using a modified Lowry method (6). GSH reductase activity based on μmoles NADPH oxidized/min/mg protein was calculated using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$. GSH peroxidase activity was calculated using a coupled reaction, whereby each μmole of NADPH oxidized by GSH reductase equals one μmole of cumene hydroperoxide reduced by GSH peroxidase. GSH-S-transferase activity was calculated as μmoles product formed/min/mg protein with an extinction coefficient of 9.6 $\mu M^{-1}cm^{-1}$.

Statistical Analysis

Accuracy and precision, two measurements of technique error, were analyzed to determine the acceptability of the Centrifichem method. Although there are several methods by which accuracy and precision can be assessed (7,8), comparison of the test (Centrifichem) method with an established reference (spectrophotometer) method was used in this study (9). Using linear regression, the results were tested to provide estimates of random error (precision) and the two components of accuracy, constant and proportional systematic error.

Results and Discussion

Protein determinations of the various tissue samples obtained by both methods were used to determine the corresponding specific activity for each enzyme. An intercept estimate of 0.101 and 0.107, and a slope estimate of 1.840 and 1.810 for spectrophotometer and Centrifichem, respectively, were obtained from linear regression analyses from standard curves of BSA. A statistical test for equality of intercepts and slopes indicated no significant difference between instruments.

Table I shows the enzyme activities measured for the lung, liver, and kidney. Prior to linear regression analysis of the data obtained from both methods, the Shapiro-Wilk W statistic (10) was used to test for normal distributions (Table I). With the exception of GSH reductase in the liver, the results indicated that the data were from normal populations.

A measure of the percent of variability explained by the linear regression model (R^2) varied from 0.66 to 0.96, with one poor fit ($R^2 = 0.26$) for GSH reductase in the lung (Table II). The

Table I. Comparison of Enzyme Activities and Results of the Normality Test

Tissue (N = 12)	GSH Enzyme	Enzyme Activity* Spectrophotometer	Enzyme Activity* Centrifichem 400	P Values for W Statistic** Spectrophotometer	P Values for W Statistic** Centrifichem 400
Lung	peroxidase	0.4560±0.0105	0.4953±0.0098	0.65	0.77
	transferase	0.0237±0.0006	0.0226±0.0006	0.49	0.31
	reductase	0.0178±0.0004	0.0210±0.0004	0.40	0.58
Liver	peroxidase	2.3200±0.0369	2.4054±0.0299	0.07	0.75
	transferase	1.3588±0.0515	1.4422±0.0568	0.30	0.38
	reductase	0.0943±0.0079	0.1107±0.0086	0.01	0.01
Kidney	peroxidase	1.9480±0.0613	2.2160±0.0625	0.19	0.09
	transferase	0.1236±0.0032	0.1348±0.0044	0.50	0.45
	reductase	0.1965±0.0060	0.1975±0.0059	0.28	0.06

*GSH reductase and peroxidase data are μmol NADPH oxidized/min/mg protein. GSH-S-transferase data are μmol product (2,4-dinitro-1-benzyl) glutathione/min/mg protein; all data are mean ± standard error.
**Values ◄0.05 indicate significance.

plot of the data suggested this was due to high variability rather than a non-linear relationship. A potential problem with using the R^2 statistic was its sensitivity to the range of data and, when using narrow ranges as in this study, the R^2 value would be lower. The coefficients of variation (CV), unitless measures of relative dispersion, were all small and consistent with one another. This indicated that, in spite of the large differences in activity for a particular enzyme in the lung, liver, and kidney, the same relationship between the mean and standard deviation (SD) existed for that enzyme.

GSH peroxidase in the liver samples showed evidence of both constant and proportional errors ($p◄0.05$). However, since GSH peroxidase in the other tissues was shown to be statistically free of these errors, it was possibly the result of the small number of samples.

Constant error was detected ($p◄0.05$) between the GSH reductase lung tissue comparisons; however, proportional error was absent. The kidney samples were free of these sources of bias. Analysis of GSH reductase activity in the liver tissue samples indicated no constant or proportional error. However, because of the non-normal distribution, a non-parametric test, Wilcoxin Signed ranks, was also performed (11). With this test, it was not possible to determine the source of the error, but the results indicated a significant difference between the Centrifichem and spectrophotometer readings. The test was very sensitive to signs and the Centrifichem readings were all slightly greater than the corresponding spectrophotometer readings. The fact that the regression analysis generated the appropriate parameter values ($m = 1$, $b = 0$) suggested that although a bias existed, it was probably not significant.

Glutathione-S-transferase samples, regardless of the tissue, exhibited good accuracy and precision. None of the y-intercepts were statistically different from 0 and the slope estimates did not differ statistically from 1.

Samples of varying concentrations of commercially purified GSH reductase were assayed in both systems. The estimated regression line was $y = -0.0002 + 1.03X$ with $R^2 = 0.99$. The accuracy of the Centrifichem relative to the spectrophotometer was not affected by proportional error (slope = 1.03). There was evidence of statistical constant error because the estimate of the y-intercept was different from 0; however, this number (−0.0002) was too small to be biologically meaningful.

Table II. Regression Analysis Results (Spectrophotometer vs Centrifichem 400)

Tissue (N = 12)	GSH Enzyme	CV	Constant Error Inter-cept	Constant Error P-value	Proportional Error Slope	Proportional Error P-value	Random Error Sy	R^2
Lung	peroxidase	6.32	0.13	0.09	0.80	0.21	0.018	0.73
	transferase	3.73	0.003	0.56	0.85	0.41	0.001	0.70
	reductase	5.50	0.01	0.03	0.51	0.10	0.001	0.26
Liver	peroxidase	5.60	0.87	0.03	0.66	0.05	0.063	0.66
	transferase	2.62	0.03	0.87	1.04	0.72	0.068	0.89
	reductase	4.69	0.01	0.14	1.06	0.39	0.006	0.96
Kidney	peroxidase	6.18	0.41	0.15	0.93	0.60	0.095	0.83
	transferase	4.27	0.02	0.52	1.23	0.30	0.008	0.77
	reductase	5.75	0.04	0.30	0.81	0.31	0.012	0.67

The Centrifichem 400 proved to be both statistically and biologically acceptable in measuring GSH peroxidase, GSH-S-transferase, and GSH reductase activities. The successful adaptation of these assays to the automated centrifugal analyzer indicates the utility and versatility of this instrument for biochemical assays beyond standard clinical chemistries.

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This report has been reviewed by the Health Effects Research Laboratory, U.S. EPA, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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